Interactions between human BRCA2 protein and the meiosis-specific recombinase DMC1

Tina Thorslund, Fumiko Esashi
and Stephen C West*

Clare Hall Laboratories, Cancer Research UK, London Research Institute, South Mimms, Herts, UK

Germline mutations in *BRCA2* predispose to hereditary breast cancers. BRCA2 protein regulates recombinational repair by interaction with RAD51 via a series of degenerate BRC repeat motifs encoded by exon 11 (BRCA2$_{996–2113}$), and an unrelated C-terminal domain (BRCA2$_{3265–3330}$). BRCA2 is also required for meiotic recombination. Here, we show that human BRCA2 binds the meiosis-specific recombinase DMC1 and define the primary DMC1 interaction site to a 26 amino-acid region (BRCA2$_{2386–2411}$). This region is highly conserved in BRCA2 proteins from a variety of mammalian species, but is absent in BRCA2 from *Arabidopsis thaliana, Caenorhabditis elegans,* and other eukaryotes. We demonstrate the critical importance of Phe2406, Pro2408, and Pro2409 at the conserved motif $_{2404}$KVFVPPFK$_{2411}$. This interaction domain, defined as the PhePP motif, promotes specific interactions between BRCA2 and DMC1, but not with RAD51. Thus, the RAD51 and DMC1 interaction domains on BRCA2 are distinct from each other, allowing coordinated interactions of the two recombinases with BRCA2 at meiosis. These results lead us to suggest that BRCA2 is a universal regulator of RAD51/DMC1 recombinase actions.

Introduction

The BRCA2 tumour suppressor protein is involved in maintaining the integrity of our genome by its participation in error-free double-strand break (DSB) repair via homologous recombination (HR; Venkitaraman, 2002). It does so by regulating the actions of RAD51 protein. Recombinases belonging to the RecA/RadA/RAD51 family play a key role in HR by binding to single-stranded DNA generated at the site of a DSB, to form helical nucleoprotein filaments that interact with homologous DNA and promote strand exchange (West, 2003). BRCA2 and RAD51 interact through a series of eight degenerative motifs within the exon 11 region of BRCA2, which are called the BRC repeats (Bignell *et al*, 1997; Wong

*Corresponding author. Clare Hall Laboratories, Cancer Research UK, London Research Institute, South Mimms, Herts EN6 3LD, UK. Tel.: +44 1707 625868; Fax: +44 1707 625811;
E-mail: stephen.west@cancer.org.uk

et al, 1997; Chen *et al*, 1998b). In addition, there is an unrelated RAD51-binding site located close to the C terminus of BRCA2 (Mizuta *et al*, 1997; Sharan *et al*, 1997), which is regulated in a cell cycle- and DNA damage-dependent manner (Esashi *et al*, 2005). In response to DNA damage, RAD51 localises to nuclear foci with BRCA2 (Scully *et al*, 1997; Sharan *et al*, 1997; Chen *et al*, 1998a), by a mechanism that is BRCA2-dependent (Yuan *et al*, 1999). Consistent with a role for BRCA2 in the regulation of HR, BRCA2-deficient cells are defective in DSB repair (Moynahan *et al*, 2001; Xia *et al*, 2001) and exhibit spontaneous gross chromosomal rearrangements (Yu *et al*, 2000).

HR has a dual cellular function: while it maintains the integrity of DNA in somatic cells, it creates genetic variability by directing DNA exchanges in meiotic cells that result in random combinations of alleles and traits. BRCA2 appears to play an important role in meiotic recombination in mammalian cells, as it is highly expressed during spermatogenesis in mice (Connor *et al*, 1997), and localises to meiotic chromosomes during early prophase I when homologous chromosomes undergo pairing (Chen *et al*, 1998a). Although targeted disruption of the *BRCA2* gene in mouse leads to embryonic lethality (Ludwig *et al*, 1997; Sharan *et al*, 1997; Suzuki *et al*, 1997), an analysis of the role of BRCA2 in meiosis has been made possible through the generation of a viable *Brca2* null mouse carrying a bacterial artificial chromosome containing the human *BRCA2* gene (Sharan *et al*, 2004). These mice, which exhibit low levels of expression of human BRCA2 protein in the gonads, are infertile because spermatocytes fail to progress beyond the early prophase I stage of meiosis. A role for BRCA2 in meiotic recombination has also been demonstrated in *Arabidopsis thaliana* where siRNA against BRCA2 leads to meiotic defects and partial sterility (Siaud *et al*, 2004).

In addition to RAD51, meiotic recombination requires the functions of DMC1, a meiosis-specific paralog of RAD51 (Bishop *et al*, 1992; Yoshida *et al*, 1998). Like RAD51, recombinant human DMC1 protein forms octameric rings that are capable of binding DNA and forming helical nucleoprotein filaments that catalyse strand exchange (Masson *et al*, 1999; Passy *et al*, 1999; Kinebuchi *et al*, 2004; Sehorn *et al*, 2004; Bugreev *et al*, 2005). The two recombinases colocalise to nuclear foci during meiotic recombination (Bishop, 1994; Chen *et al*, 1998a; Tarsounas *et al*, 1999). Consistent with these observations, mouse *Dmc1* knockouts are infertile due to defects in chromosome synapsis during meiotic recombination (Pittman *et al*, 1998; Yoshida *et al*, 1998). However, it is not known how these two recombinases function during meiosis, whether they play independent roles or act in concert, or how their activities are regulated and coordinated.

A potential role for BRCA2 in coordinating the activities of RAD51 and DMC1 was implied by studies showing that meiotic chromosomes from mouse spermatocytes exhibiting impaired expression of BRCA2 had reduced numbers of RAD51 and DMC1 foci (Sharan *et al*, 2004). Also, in plants the *At*BRCA2 protein has been shown to interact with *At*DMC1,

an interaction mediated by one of the four BRC repeats (BRC2) found in AtBRCA2 (Siaud *et al*, 2004; Dray *et al*, 2006). To date, however, there is no information relating to interactions between mammalian BRCA2 and DMC1 proteins.

In this study, we define the interactions between human BRCA2 and DMC1. Surprisingly, we found that the primary DMC1 interaction domain of BRCA2 maps to a region of BRCA2 that is distinct from the BRC repeats that facilitate interactions with RAD51. These results provide the first insight into understanding the coordinating role that BRCA2 provides in mammalian meiotic recombination.
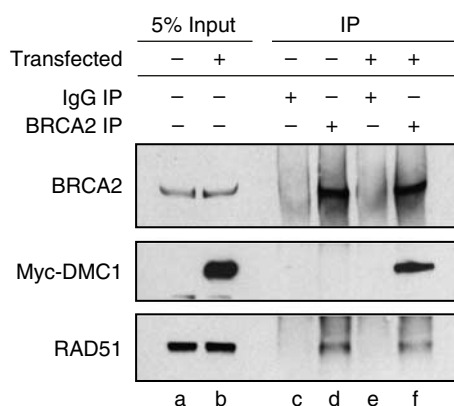
Results

In vivo interactions of BRCA2 with DMC1

The interaction of human BRCA2 and DMC1 *in vivo* was investigated by using rabbit polyclonal antibodies to immunoprecipitate BRCA2 complexes from extracts prepared from human 293T cells transiently expressing Myc-tagged DMC1. The BRCA2 pull-downs were analysed by Western blotting using mouse monoclonal antibodies specific for BRCA2 (Figure 1, top panel), Myc-DMC1 (middle panel), and RAD51 (lower panel). We found that the BRCA2-specific antibodies pulled down both DMC1 and RAD51 (lane f), whereas IgG control reactions failed to pull down either of these proteins (lane e). Control reactions, using extracts from mock-transfected 293T cells, rather than Myc-DMC1-transfected 293T cells, showed the absence of DMC1 from the BRCA2-RAD51 immunoprecipitates, as expected since DMC1 is not normally expressed in somatic cells (lane d). Interestingly, the amounts of endogenous RAD51 present in the BRCA2 immunoprecipitates from Myc-DMC1 or mock-transfected cells were similar, indicating that while DMC1 can interact with BRCA2 *in vivo*, its overexpression does not significantly affect the binding of RAD51 to BRCA2.

Mapping the DMC1 interaction sites in BRCA2

Studies of the interaction between BRCA2 and DMC1 in *A. thaliana* indicate that DMC1, like RAD51, interacts with BRCA2 through the BRC repeat sequences (Dray *et al*,

2006). To map the interaction domains between the human BRCA2 and DMC1 proteins, we used a series of nine overlapping glutathione *S*-transferase (GST) fusion proteins, designated B2-1 to B2-9 (Lee *et al*, 2004), that span the entire coding region of BRCA2 (Figure 2A). Lysates from *Escherichia coli* expressing each of the nine GST fusion proteins (Figure 2B, lanes b–j), and GST alone (lane a), were incubated with glutathione beads, and purified recombinant human DMC1 or RAD51. Complexes bound to the beads were analysed by Western blotting for either DMC1 (Figure 2B, top panel) or RAD51 (Figure 2B, middle panel). Similar amounts of GST-tagged BRCA2 fragments were observed in each pull-down (Figure 2B, bottom panel). As reported previously (Esashi *et al*, 2005), RAD51 interacted specifically with B2-3 (lane d) and B2-4 (lane e), the fragments containing BRC repeats 1–5, and with B2-9 (lane j), which corresponds to the C-terminal region of BRCA2. In the case of DMC1, however, we failed to observe interactions between DMC1 and any of the BRCA2 GST fragments containing BRC repeats (Figure 2B, top panel, lanes d–f). Instead, we found that DMC1 interacted specifically with B2-6 (lane g) and with the C-terminal region B2-9 (lane j).

A region of BRCA2 that interacts with DMC1 but not RAD51

Presently, there is very little information relating to the function of the region of BRCA2 defined by B2-6 (amino
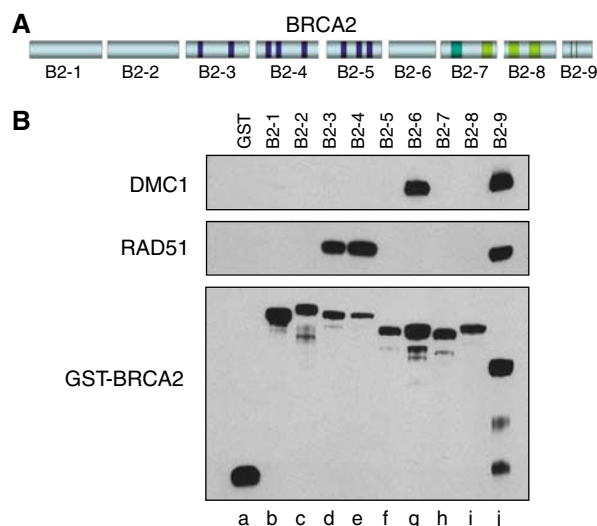
Figure 2 Interactions of BRCA2 with RAD51 and DMC1. (**A**) Schematic representation of human BRCA2 protein divided into nine GST-tagged fusion proteins. BRC repeats 1–8 (dark blue), the helical domain (green), OB folds (yellow), and nuclear localisation signals (black) are indicated. The boundaries of the nine fragments are as follows: B2-1 is BRCA2$_{1-454}$; B2-2 is BRCA2$_{420-906}$; B2-3 is BRCA2$_{885-1362}$ and contains BRC1 and BRC2; B2-4 is BRCA2$_{1338-1781}$ and contains BRC3, BRC4, and BRC5; B2-5 is BRCA2$_{1744-2115}$ and contains BRC6, BRC7, and BRC8; B2-6 is BRCA2$_{2106-2472}$; B2-7 is BRCA2$_{2438-2824}$ and contains the helical domain and one OB fold; B2-8 is BRCA2$_{2780-3197}$ and contains two OB folds; B2-9 is BRCA2$_{3189-3418}$ and contains two nuclear localisation signals. (**B**) Interactions of recombinant DMC1 or RAD51 with the nine GST-tagged BRCA2 fusion proteins. Lysates of *E. coli* cells expressing the indicated GST fusions were mixed with glutathione beads, and purified human RAD51 or DMC1 was subsequently added. After extensive washing, complexes bound to beads were analysed by Western probing for DMC1 (upper panel), RAD51 (middle panel), or the BRCA2 fragments (lower panel).


Figure 1 DMC1 interacts with BRCA2 in human cells. Human 293T cells were either mock-transfected (input, lane a) or transfected with Myc-DMC1 (input, lane b). Post-transfection, cells were harvested, lysed, and protein complexes were immunoprecipitated with polyclonal BRCA2 antibodies or nonspecific rabbit IgG, as indicated. The immunoprecipitates were then analysed by Western blotting for BRCA2 (upper panel), Myc-DMC1 (middle panel), or RAD51 (lower panel).

acids 2106–2472). To map the DMC1 interaction domain in greater detail, we subdivided the region into a series of smaller GST fusion proteins, depicted in Figure 3A, designated B2-6.1 to B2-6.5. These fusion proteins were then expressed and used in DMC1 pull-down assays (Figure 3B). We found that DMC1 interacted strongly with B2-6.5 (Figure 3B, lane g), corresponding to $BRCA2_{2340-2472}$, but did not interact with any of the other fusion proteins (lanes c–f).

To ensure that the B2-6.5 region of BRCA2 interacts specifically with DMC1, competition experiments were carried out between RAD51 and DMC1 for binding to B2-6.5. We found that a 10-fold excess of purified RAD51 had no effect on the interaction between DMC1 and B2-6.5 (Figure 3C, compare lanes a–d). In these reactions, RAD51 could not be detected in the B2-6.5 pull-down by Western blotting (data not shown). These experiments indicate that there are no direct interactions between the two recombinases and show that $BRCA2_{2340-2472}$ contains a novel domain that binds specifically to DMC1 but does not interact with RAD51.

The interaction of this novel BRCA2 domain with DMC1 was confirmed by yeast two-hybrid analysis, a system used previously to evaluate BRCA2-RAD51 interactions (Wong

et al, 1997; Chen *et al*, 1998b). It was also the primary method used to map interactions between a BRC repeat of *At*BRCA2 and *At*DMC1 (Dray *et al*, 2006). For the yeast two-hybrid analysis, DMC1 and RAD51 were fused to the GAL4 activation domain, while a variety of BRCA2 fragments were fused to the GAL4 DNA-binding domain. We used the eight BRC repeats, B2-6 and B2-6.5 (both identified in the above GST pull-down experiments to interact specifically with DMC1), and the C-terminal BRCA2 fragments B2-9 and TR2 (previously shown to be the C-terminal RAD51 interaction domain (Esashi *et al*, 2005)). We also expressed a large BRCA2 fragment, $BRCA2_{996-2113}$, which contains all eight BRC repeats. We found that BRCA2-DMC1 interactions were observed with the two constructs containing the newly identified DMC1 interaction domain $BRCA2_{2340-2472}$ (Figure 4, B2-6.5 and B2-6). Neither of these BRCA2 fragments resulted in growth when combined with RAD51. These results confirm the finding that $BRCA2_{2340-2472}$ contains a domain that interacts specifically with DMC1.

As expected, RAD51 was found to interact with the C-terminal part of BRCA2, several of the individual BRC repeats, and with the $BRCA2_{996-2113}$ fragment containing all BRC repeats. In the two-hybrid assay, we also observed an interaction between DMC1 and BRC repeat 4 (BRC4), but this interaction was relatively weak in comparison with those of B2-6 or B2-6.5, as indicated by the lack of growth in the absence of uracil (−LTU). Importantly, growth was not observed when DMC1 was expressed with $BRCA2_{996-2113}$, which contains all eight BRC repeats. The weakness of the BRC4-DMC1 interaction, coupled with the absence of similar interactions in the GST pull-down experiments, may question the physiological relevance of this interaction.

### Interactions of RAD51 and DMC1 with the C-terminal region of BRCA2

The two-hybrid analysis confirmed interactions between the C-terminal region of BRCA2 (TR2 and B2-9) with RAD51, but failed to support the pull-down experiments indicating an interaction of this region with DMC1. Further experiments were therefore carried out to establish that both RAD51 and DMC1 could interact with this domain of BRCA2. First, we used three small GST-tagged BRCA2 fragments, TR1, TR2, and TR3 (Figure 5A), to map the interaction of DMC1 with the C-terminal region of BRCA2 in greater detail. GST pull-downs showed that both DMC1 and RAD51 bind to the TR2 fusion protein ($BRCA2_{3265-3330}$), but not to either TR1 ($BRCA2_{3189-3274}$) or TR3 ($BRCA2_{3325-3418}$) (Figure 5B). Next, we carried out competition experiments to determine which of the two proteins exhibited the highest binding affinity for this site in BRCA2. For this analysis, TR2 bound to glutathione beads was mixed with constant amounts of DMC1 and increasing amounts of RAD51 and, after extensive washing, the complexes bound to beads were analysed by Western blotting. We found that the presence of RAD51, even at submolar concentrations compared with DMC1, efficiently competed away the binding of DMC1 to the TR2 region of BRCA2 (Figure 5C, lanes a–d). In contrast, DMC1 failed to compete with RAD51 for interaction with TR2 (lanes e–h). These results indicate that the DMC1-TR2 interaction is relatively weak compared to RAD51-TR2 complex formation, helping to explain why these interactions were not seen in the yeast two-hybrid studies.
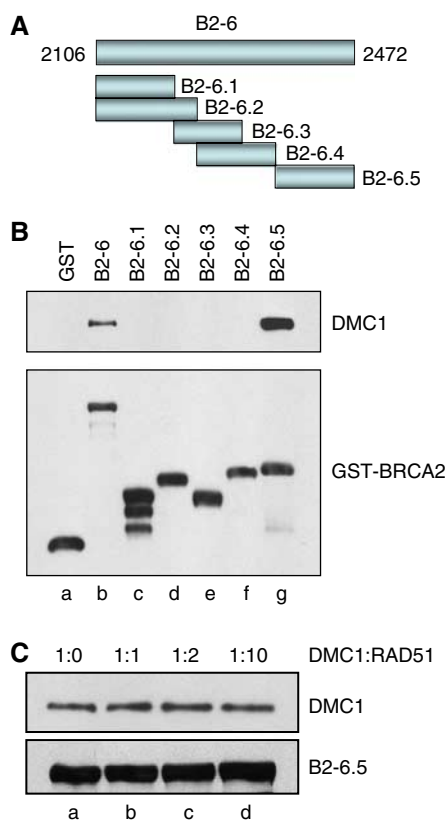
Figure 3 Identification of a DMC1 interaction domain in an internal region of BRCA2. (**A**) Schematic representation of BRCA2 B2-6, subdivided into five smaller GST fusion proteins. B2-6.1 is $BRCA2_{2106-2190}$; B2-6.2 is $BRCA2_{2106-2218}$; B2-6.3 is $BRCA2_{2190-2260}$; B2-6.4 is $BRCA2_{2218-2340}$; B2-6.5 is $BRCA2_{2340-2472}$. (**B**) The indicated GST fusions were expressed in *E. coli* as described in Figure 2 legend and their ability to bind purified DMC1 was analysed as described. (**C**) Competition assay to analyse interactions between DMC1 or RAD51 and B2-6.5 fusion protein. B2-6.5 was mixed with DMC1 (200 ng) and increasing amounts of RAD51 (0, 200, 400, or 2 µg, respectively). Proteins bound to glutathione beads were analysed by Western blotting.
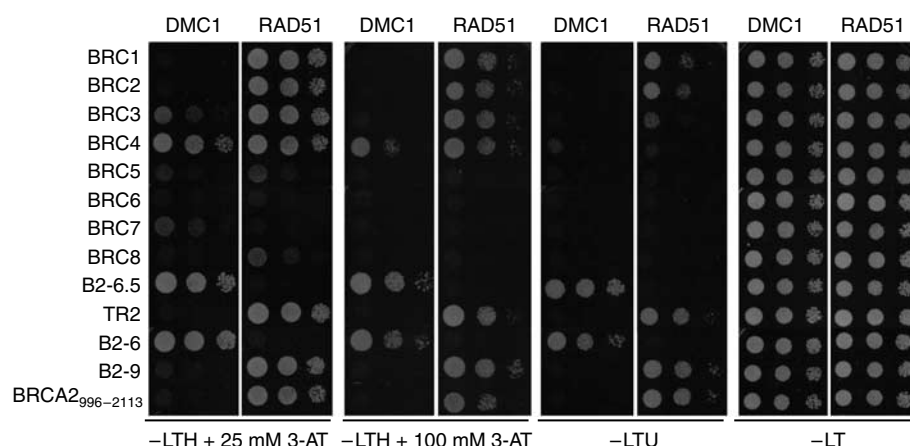
Figure 4 Yeast two-hybrid analysis of interactions between DMC1 or RAD51 and a series of BRCA2 fragments. Two-hybrid analysis was carried out as described in Methods, and diluted cells were plated on permissive −Leu/−Trp plates (−LT), or selection plates: −Leu/−Trp/−His + 25 mM 3-AT (−LTH + 25 mM 3-AT); −Leu/−Trp/−His + 100 mM 3-AT (−LTH + 100 mM 3-AT); or −Leu/−Trp/−Ura (−LTU). The BRCA2 fragment boundaries are as follows: BRC1 (BRCA2$_{996-1084}$); BRC2 (BRCA2$_{1206-1274}$); BRC3 (BRCA2$_{1415-1483}$); BRC4 (BRCA2$_{1511-1579}$); BRC5 (BRCA2$_{1658-1726}$); BRC6 (BRCA2$_{1831-1899}$); BRC7 (BRCA2$_{1965-2033}$); BRC8 (BRCA2$_{2045-2113}$); B2-6 (BRCA2$_{2106-2472}$); B2-6.5 (BRCA2$_{2340-2472}$); B2-9 (BRCA2$_{3189-3418}$); TR2 (BRCA2$_{3265-3330}$). A large BRCA2 fragment, BRCA2$_{996-2113}$, containing all eight BRC repeats was also analysed.
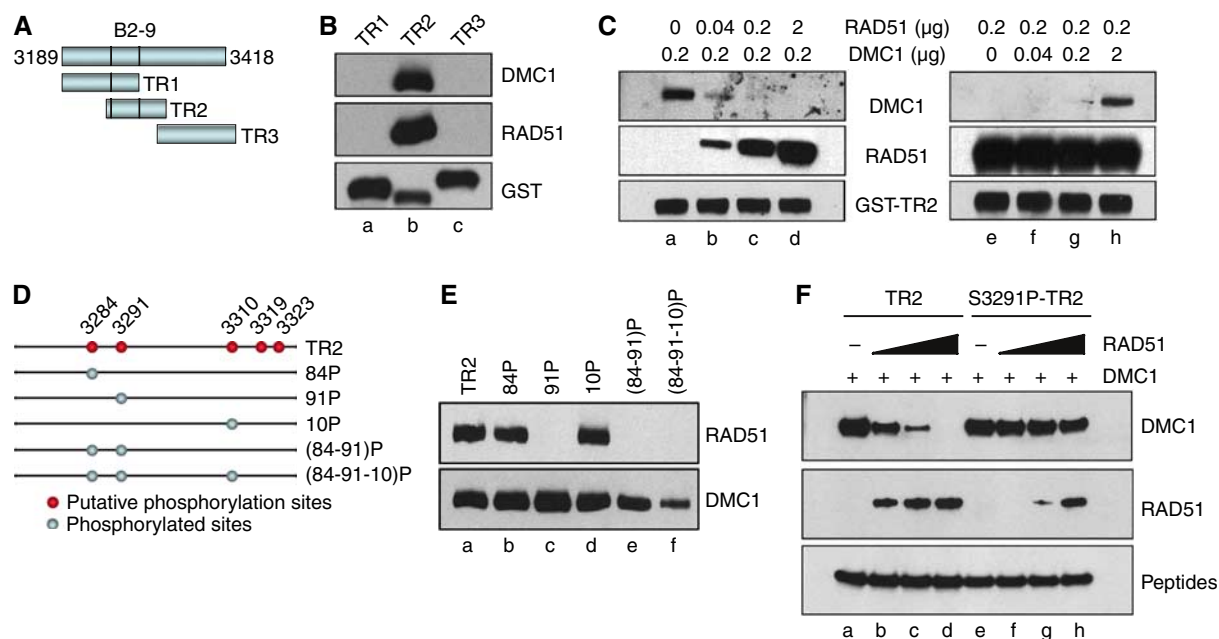
Figure 5 Characterisation of interactions between DMC1 and the C-terminal region of BRCA2. (**A**) Schematic representation of BRCA2 B2-9, subdivided into three smaller GST fusion proteins. TR1 is BRCA2$_{3189-3274}$ and contains one nuclear localisation signal (black); TR2 is BRCA2$_{3265-3330}$ and contains both nuclear localisation signals; TR3 is BRCA2$_{3325-3418}$. (**B**) Interaction of DMC1 or RAD51 with the three GST-tagged BRCA2 TR-fragments. Complexes bound to glutathione beads were analysed by Western probing for DMC1, RAD51, and BRCA2 fragments, as described in Figure 2b. (**C**) Competition between RAD51 and DMC1 for TR2 binding. TR2 was mixed with DMC1 and increasing amounts of RAD51 (lanes a–d), or RAD51 was mixed with increasing amounts of DMC1 (lanes e–h). Complexes bound to glutathione beads were analysed by Western blotting. (**D**) Schematic representation of the TR2 region of BRCA2. Red circles correspond to putative phosphorylation sites and blue circles to sites that have been phosphorylated in synthetic biotinylated peptides. The S3291 is known to be phosphorylated *in vivo*. (**E**) Interactions between RAD51 or DMC1 and the series of phosphopeptides shown in (d). Synthetic biotinylated phosphopeptides (300 ng) were mixed with either DMC1 or RAD51 (60 ng), and complexes bound to Streptavidin beads were analysed by Western blotting. (**F**) Effect of S3291 phosphorylation on the competition between RAD51 and DMC1 for TR2. TR2 peptide or S3291Phospho-TR2 peptide (300 ng) was mixed with DMC1 (60 ng) and increasing amounts of RAD51 (0, 30, 60, or 180 ng). Complexes bound to Streptavidin beads were analysed by Western blotting. Biotinylated peptides were visualised by HRP-conjugated Streptavidin.

Previously, it was shown that CDK-dependent phosphorylation of serine 3291 in the TR2 region of BRCA2 inhibits RAD51 interaction with this site and that this modification may serve as a regulatory mechanism for recombinational repair (Esashi *et al*, 2005). We therefore investigated the

effect of TR2 phosphorylation on the binding of DMC1 to this region. Using biotinylated TR2 peptides phosphorylated at S3291, or a series of other control sites (Figure 5D), we found that peptides containing phosphorylated S3291 failed to bind RAD51 but were capable of interacting normally with

DMC1 (Figure 5E, lanes c, e and f). These results were confirmed by competition studies in which biotinylated peptides corresponding to TR2, unphosphorylated, or phosphorylated at S3291, were incubated with DMC1 in the presence of increasing amounts of RAD51. As observed with TR2, we found that RAD51 could easily compete for DMC1 binding to TR2 (Figure 5F, lanes a–d), whereas it failed as a competitor with S3291-phosphorylated TR2 (lanes e–h). Thus, the phosphorylation of S3291 can modulate interactions with RAD51, but not DMC1, and may provide a mechanism for the differential regulation of BRCA2-RAD51 and BRCA2-DMC1 interactions.

Identification of a conserved domain in BRCA2, essential for DMC1 interaction

Since yeast two-hybrid and GST pull-down analyses had both confirmed the presence of a specific DMC1 interacting region in B2-6.5. (BRCA2$_{2340-2472}$), we wished to define this domain in greater detail. First, we generated four overlapping biotinylated synthetic peptides covering the region (Figure 6A, IR1–IR4), and used them in streptavidin pull-down experiments to show that the DMC1 interaction domain was contained within IR2 (BRCA2$_{2350-2417}$) and IR3 (BRCA2$_{2371-2438}$) (Figure 6B). Second, to narrow down this interaction domain even further, we synthesised a 30-mer peptide array that covered the entire BRCA2$_{2350-2417}$ region. On this membrane, which was subsequently probed with DMC1, each peptide overlapped the next by 29 amino acids, leading to a change of just one amino acid per peptide. We found that DMC1 interacted strongly with six to seven consecutive peptides, all containing the region corresponding to BRCA2$_{2386-2411}$ (Figure 6C, lower panel). This region is highly conserved in mammalian BRCA2 from a variety of species (Figure 6D), and is also found in chicken BRCA2. In contrast, the region has diverged in the BRCA2 homologues found in *A. thaliana*, *Caenorhabditis elegans*, *Ustilago maydis*, and *Trypanosoma cruzi*.

Finally, to define precisely which amino acids are essential for the BRCA2-DMC1 interaction, we generated a mutant peptide array of 30-mer peptides containing the conserved region of BRCA2 important for DMC1 interaction. In this array, the 30 amino acids corresponding to BRCA2$_{2382-2411}$ were sequentially substituted (one at a time) by the 20 common amino acids (each substitute is indicated at the right of the membrane in Figure 6E). Hence, on this membrane, the first phenylalanine (F2382) is substituted in the first column, in the second column the tyrosine (Y2383) has been substituted, and so on. When this membrane was probed for DMC1 interaction, we observed that substitution of Phe2406 with any amino acid, except the two other aromatic amino acids, abolished interaction with DMC1. Similarly, substitution of Pro2408 or Pro2409 led to a loss of interaction with DMC1. All three amino acids were found to be located in the highly conserved $_{2404}$KVFVPPFK$_{2411}$ region of BRCA2.

Discussion

In this work, we have demonstrated that human BRCA2 is capable of binding the meiosis-specific recombinase DMC1 via two different binding sites. The first is located at the C terminus of BRCA2 and corresponds to the region also
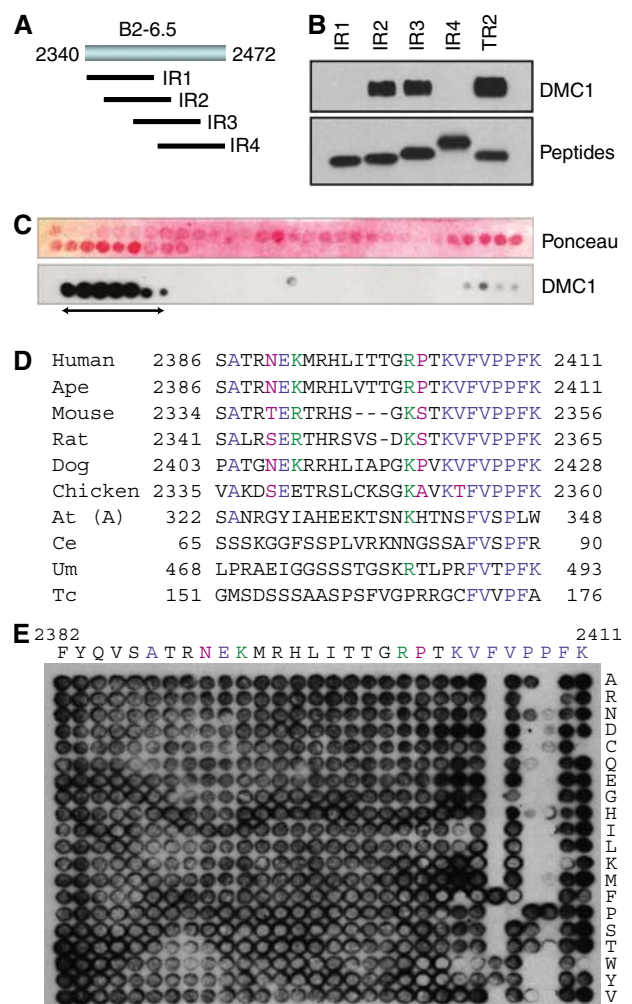
Figure 6 Identification of the DMC1 interacting domain PhePP. (**A**) Schematic representation of four overlapping peptides corresponding to B2-6.5. IR1 is BRCA2$_{2340-2407}$; IR2 is BRCA2$_{2350-2417}$; IR3 is BRCA2$_{2371-2438}$; IR4 is BRCA2$_{2405-2472}$. (**B**) Interactions between the biotinylated IR peptides (300 ng) and DMC1 (60 ng) were assayed by streptavidin pull-downs and detected by Western blotting. Biotinylated TR2 was used as a control. (**C**) Peptide array with overlapping 30 mers of BRCA2$_{2350-2417}$ spotted on a membrane (see text for detail), probed with DMC1, and analysed by Western blotting. Subsequently, the membrane was stained with Ponceau to reveal the exact location of the spotted peptides. (**D**) Sequence alignment of the PhePP motif BRCA2 from a variety of organisms. At (A): *A. thaliana* BRCA2 homologue A. Ce: *C. elegans*. Um: *Ustilago maydis*. Tc: *Trypanosoma cruzi*. Amino acids indicated in blue are fully conserved in mammalian species, green indicate conservation of strong groups, whereas purple indicates conservation of weaker groups. (**E**) Analysis of essential amino acids required for interactions between BRCA2$_{2382-2411}$ and DMC1. The amino-acid substitution peptide array contains 600 30-amino acid long peptides spotted onto a membrane. Each peptide has one amino acid of BRCA2$_{2382-2411}$ replaced with one of the 20 common amino acids (see text for details). The membrane was probed with DMC1 followed by Western blotting.

bound by RAD51 protein. The second, however, represents the primary DMC1 interaction domain located within the relatively uncharacterised central part of BRCA2, contained within B2-6. Using yeast two-hybrid assays, pull-down experiments, and peptide arrays, we defined this interaction domain to the region corresponding to BRCA2$_{2386-2411}$. Within this region, we showed the critical importance of

Phe2406, Pro2408, and Pro2409 located in the conserved motif KVFVPPFK, and for simplicity will refer to this DMC1 interaction domain as the PhePP motif.

The PhePP motif of BRCA2 interacts specifically with DMC1, and not with RAD51. The importance of the region is indicated by its conservation in different mammalian species and in chicken BRCA2. This DMC1 interaction domain, however, is divergent in BRCA2 homologues from other eukaryotes such as *A. thaliana* and *C. elegans*. When our studies were initiated, we were surprised to identify this novel interaction domain, as it was known that *At*BRCA2 and *At*DMC1 can interact through one of the four degenerate plant BRC repeats (Dray *et al*, 2006). However, modelling studies based on the crystal structure of a RAD51-BRC4 fusion protein suggest that DMC1 has polar or charged residues at the hydrophobic RAD51-binding patch for BRC4, which is likely to preclude its interaction with this BRC repeat (Pellegrini *et al*, 2002; Lo *et al*, 2003). It might also be significant that the *At*BRCA2 proteins (there are two *At*BRCA2 isoforms at 1151 or 1155 amino acids) are considerably shorter than the human counterpart (3418 amino acids), such that two of the plant BRC repeats (BRC4 and BRC2, respectively) have evolved independently to interact with either RAD51 or DMC1 (Dray *et al*, 2006). It remains to be established whether the diverged PhePP motif in *A. thaliana* can interact with AtDMC1. The divergence of the PhePP domain in *C. elegans* is less surprising given the absence of DMC1 in this organism, which promotes meiotic recombination through the high-level expression of RAD51 alone (Takanami *et al*, 2000).

In addition to the specific DMC1 interaction domain in BRCA2, we found that DMC1, like RAD51, could interact with the C-terminal region of BRCA2 known as TR2 (BRCA2$_{3265-3330}$). In comparison to the RAD51-TR2 interaction, the association of DMC1 with TR2 appears to be relatively weak, and it was found that RAD51 could easily compete with DMC1 for the C-terminal interaction site. However, it is known that interactions between RAD51 and the C terminus of BRCA2 are blocked by S3291 phosphorylation, a modification event that has little or no impact upon DMC1 binding. Thus, circumstances might exist that would favour DMC1, rather than RAD51, interaction with the BRCA2 C terminus. Our attempts to demonstrate phosphorylation of S3291 during meiotic recombination, which might favour interactions with DMC1 have so far proven to be unsuccessful, so the relevance of this potential binding switch upon phosphorylation is presently unknown. However, the lack of meiotic defects in mice harbouring deletions of the BRCA2 C terminus may well indicate that interactions between the BRCA2 C-terminal region and RAD51/DMC1 may not be so critical for the functions of BRCA2 during meiotic recombination (Atanassov *et al*, 2005).

The precise role played by BRCA2 in regulating homologous recombinational repair of DNA DSBs has yet to be fully defined. However, we are now beginning to understand the key role played by this protein in binding RAD51 and facilitating its relocalisation in somatic cells in response to DNA breaks. A similar situation exists in meiotic cells, as RAD51 and DMC1 colocalise to Spo11-induced DSBs during meiotic recombination (Bishop, 1994; Tarsounas *et al*, 1999). Our observations of a novel DMC1 interaction site at BRCA2$_{2386-2411}$, located away from the BRC repeats that mediate the primary inter-

actions with RAD51, lead us to speculate that BRCA2 is capable of coordinately binding RAD51 and DMC1 to form a BRCA2-RAD51-DMC1 complex, which may facilitate the localisation of both recombinases to DNA break sites at the initiation of meiotic recombination. There, as in mitotic cells, it is likely that BRCA2 may participate in nucleoprotein filament formation leading to subsequent repair.

Materials and methods

Plasmids
The GST-tagged BRCA2 constructs B2-1 to B2-9 (Lee *et al*, 2004) and TR1–TR3 (Esashi *et al*, 2005) have been described previously. The GST-tagged BRCA2 constructs B2-6.1 to B2-6.5 were cloned into the *Sal*I/*Not*I sites of pGEX-4T3 (Amersham) using PCR amplification. The mammalian expression vector pMyc-DMC1 was created by PCR amplification and subsequent insertion of the DMC1 sequence at the *Bam*HI/*Sal*I sites in the pCMV-Tag3 vector (Stratagene). The yeast two-hybrid plasmids were all cloned by PCR into the Gateway pDONR221 vector (Invitrogen) and subsequently transferred into pDEST22 (RAD51 and DMC1), or pDEST32 (all the BRCA2 constructs) (Invitrogen). All PCR-amplified sequences were verified by sequencing.

Proteins and peptides
Recombinant His$_{10}$-DMC1 and RAD51 were prepared as described (Baumann *et al*, 1997; Masson *et al*, 1999). Peptides were made by solid-phase synthesis and purified by high-performance liquid chromatography, and their sequences were verified by mass spectroscopy. All peptides contained an N-terminal biotin group with an aminohexanoic spacer. GST-tagged BRCA2 constructs were expressed in *E. coli* strain BL21 RIL codon plus (Stratagene) by IPTG induction (0.1 mM) for 2 h at 18°C.

In vivo protein interactions
Human 293T cells were cultured in E4 medium supplemented with 10% FBS and antibiotics. Transfection with pMyc-DMC1 was performed using Fugene 6 (Roche) according to the manufacturer's instructions. Twenty-four hours post-transfection, cell were collected and lysed using a modified RIPA buffer (50 mM Tris–HCl, pH 7.5, 0.2% NP-40, 0.1% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na$_3$VO$_4$, 1 mM NaF, and Complete Protease Inhibitor Cocktail (Roche)). The lysates were added to 20 μl protein A magnetic beads (Invitrogen), which had been preincubated with 20 μg nonspecific rabbit IgG (Santa Cruz Biotechnology) or anti-BRCA2 rabbit polyclonal antibodies (Ab-2, Calbiochem) in RIPA buffer containing 2 mg/ml BSA. After incubation at 4°C, the beads were washed extensively, boiled in SDS loading buffer, and the precipitates analysed by Western blotting using either BRCA2 mouse monoclonal antibodies (Ab-1, Calbiochem), c-Myc mouse monoclonal antibodies (9E10, CRUK), or RAD51 monoclonal antibodies (14B4, Abcam).

In vitro GST pull-downs
E. coli cells expressing BRCA2 fragments were lysed in IP buffer (50 mM Tris–HCl, pH 7.5, 250 mM KCl, 5% glycerol, 0.5% NP-40, 2 mM EDTA, 1 mM DTT, 1 mM Pefabloc SC, Complete Protease Inhibitor Cocktail (Roche), and 25 U/ml Benzonase Nuclease). Saturating amounts of lysates were mixed with 15 μl glutathione Sepharose 4B beads (Amersham), and incubated with 200 ng DMC1 or RAD51 in IP buffer containing 2 mg/ml BSA. After incubation and extensive washing with IP buffer, the precipitates were analysed by Western blotting using HRP-conjugated GST mouse monoclonal antibodies (B14, Santa Cruz Biotechnology), 6 × His mouse monoclonal antibodies (Clontech) for detection of DMC1, and RAD51 monoclonal antibodies (14B4, Abcam). For DMC1/RAD51 competition, the procedure was as above, adding 200 ng DMC1 or RAD51 alone or in combination with the designated amounts of competitor.

In vitro peptide interaction studies
Biotinylated peptides (300 ng) were mixed with 6 μl magnetic Streptavidin Beads (Invitrogen) in IP buffer containing 2 mg/ml BSA

and washed, before addition of 60 ng DMC1 or RAD51 in IP buffer containing 2 mg/ml BSA. After incubation and extensive washing with IP buffer, the precipitates were analysed by Western blotting using HRP-conjugated Streptavidin for detection of the biotinylated peptides (Abcam), $6 \times$ His mouse monoclonal antibodies (Clontech) for detection of DMC1, and RAD51 monoclonal antibodies (14B4, Abcam). For DMC1/RAD51 competition experiments, the procedure was similar to that described except that the reactions contain 60 ng DMC1 alone or in combination with the designated amounts of RAD51.

Peptide arrays

For the peptide array studies, peptides were synthesised and spotted onto cellulose membranes. The membrane used in Figure 6C contained peptides, 30 amino acids in length, corresponding to BRCA2$_{2350-2417}$. The first peptide in this array corresponds to BRCA2$_{2350-2379}$, the next to BRCA2$_{2351-2380}$, the third to BRCA2$_{2352-2381}$, and so on. The membrane was activated in 50% methanol before blocking in 5% milk in TBS + 0.1% Tween 20 (TBS-T). The membrane was then incubated with His-tagged DMC1 in 5% milk in TBS-T overnight, and bound DMC1 was detected by incubation with anti-His monoclonal antibodies and chemiluminescence. Subsequently, the membrane was stained with Ponceau, to reveal the exact location of each peptide. The mutant peptide array in Figure 6E was generated by synthesising 600 peptides corresponding to BRCA2$_{2382-2411}$, where each peptide sequentially had one of the amino acids substituted with each of the 20 common amino acids. The membrane was organised so that each of the 30 columns had the same amino acids in the 30-mer substituted. Each of the 20 rows on the membrane contains the identical amino-acid substitution.

Yeast two-hybrid assays

The ProQuest Two-Hybrid System (Invitrogen) was used for the analysis according to the manufacturer's instructions. DMC1 and RAD51 were fused to the GAL4 activation domain, while the BRCA2 fragments were fused to the GAL4 DNA-binding domain. After transforming the MaV203 strain with the different combinations of constructs by the lithium-acetate method, colonies were picked and grown in SD-medium minus Leu and Trp (Clontech) to stationary phase. The cells were then diluted and 10 000, 1000, or 100 cells, respectively, were plated onto selective plates, which were subsequently incubated at 30°C for 3 days. Interactions were assessed by analysing transcription of two different reporter genes, *URA3* and *HIS3*, which resulted in grown on agar plates lacking uracil and histidine, respectively. Furthermore, on plates lacking histidine, two different concentrations of the *HIS3* inhibitor 3-Amino-1,2,4-triazole (3-AT) was used, resulting in different stringency of these plates. Control experiments showed that growth was not observed on selective plates when the cells were transfected either with the GAL4 activation- or with DNA binding-domains.

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